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Using Theoretical Correction Factors for Quantitative Analysis of Sterols and Sterol Concentrates

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Abstract A common detector for the analysis of sterols and sterol concentrates by gas chromatography is the flame ionization detector (FID). The detector measures the response of ions from a molecule as it is pyrolyzed in a hydrogen flame. The response is relative to the number of hydrogen and carbon atoms in a molecule and it gives different responses for the same amounts of different analytes. Theoretical correction factors (TCF) can be used to account for these differences during data analysis and are based upon the number of active carbons in a molecule and can be calculated from known structures. TCF have been used to provide a more reproducible approach to the analysis of sterol concentrates in raw materials. Use of empirical correction factors (ECF) requires determination of relative responses for each analyte and also a high purity standard (and determination of that purity), which can be expensive or difficult to obtain for the more uncommon sterols. Experiments have been conducted to determine ECF for Ergost-5-en-3-ol, (3β,24R), commonly known as campesterol, Stigmasta-5,22-dien-3-ol, (3β,22E), commonly known as stigmasterol, and Stigmast-5-en-3-ol, (3β), commonly known as β-sitosterol and compared to the calculated TCF for these same sterols. The experiments evaluated differences in concentration, standard purity, and injection technique and found that all three factors had an effect on the determination of ECF. It was also found that cool on column injections gave more accurate ECF values and are preferable for quantitative analysis of sterols.

Keywords Theoretical correction factors (TCF) · Sterols · Gas chromatography · Flame ionization detection · Data analysis

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Introduction

The idea of using theoretical response factors in gas chromatography (GC) analysis coupled to flame ionization detection (FID) dates back to the late 1950s when McWilliam and Dewar [1] found when working with hydrocarbons that the relative molar response of an FID appeared to be proportional to the number of carbons in the chain. Some years later Ettre and Kabot [2] conducted an experiment that showed the relative FID response of fatty acid methyl esters (FAME) is linearly proportional to the carbon number in saturated FAMEs. Ackman and Sipos [3] shortly followed with the first proposal to use theoretical correction factors (TCF) in the analysis of saturated FAME. Bannon et al. [4] later describe a technique for using TCF in the analysis of unsaturated FAMEs. TCF continue to be used for the analysis of FAME and are found in the official AOCS method for the analysis of *cis*-, *trans*-, saturated, monounsaturated and polyunsaturated fatty acids in vegetable oils [5]. The primary benefits of TCF are they can improve speed, accuracy, and precision of analyses especially for complex mixtures of analytes where pure standards may be difficult or impossible to find. This study focused on using the three major vegetable oil sterols; Ergost-5-en-3-ol, (3β,24R), commonly known as campesterol, Stigmasta-5,22-dien-3-ol, (3β,22E), commonly known as stigmasterol, and Stigmast-5-en-3-ol, (3β),

commonly known as β-sitosterol. These sterols will be referred to by their common names throughout the remainder of the article.

An accurate determination of all the sterols in a sample can be very important when dealing with sterol concentrates, which can contain 15 or more different types of sterols. In sterol concentrates obtained from vegetable oils the three major sterols are campesterol, stigmasterol, and β-sitosterol, which contribute approximately 90% of the total sterol content (depending upon the source) with the remaining 10% coming from lower amounts of less common sterols. While TCF can be used in all sterol analysis their significance is greater in the sterol concentrates where sterols make up from 35 to 95% of total sample mass. The use of TCF alleviates the need to purchase standards, develop calibration curves and eliminates response differences between labs. In addition, TCF can be used for analysis of compounds that do not have high quality analytical standards available. This work demonstrates that the use of TCF can be applied to other classes of hydrocarbons that have not been previously reported and indicates their applicability may extend even further beyond hydrocarbons and fatty acid methyl esters (FAME).

Determination of accurate sterol amounts is critical in minimizing costly over formulation. It is important to be able to measure active ingredients such as sterols accurately to ensure that a formulation will meet requisite health claims (0.8 g/day for sterols and 1.3/day steryl esters (SEs)) [6]. Some common practices used in the quantitative analysis of sterols in raw materials or consumer products by gas chromatography (GC) involve creating calibration curves using pure sterols or calculating an empirical correction factor using a single pure standard and applying that ECF to all sterols in a sample [7]. Anytime a standard is used in developing a response factor it is important to determine the purity of the standard being used to accurately determine the actual amount of material being weighed. Additionally, each of these approaches have problems that can effect the accuracy, precision, and efficiency of the measurement. Using external standards to create a calibration curve can be very accurate if pure standards are readily available, which is not the case for many sterols commonly found in vegetable oils. In addition, generating calibration curves requires more preparation and analysis time, which can affect the efficiency of a lab. There are also errors associated with preparation of calibration curves, which can be propagated through the analysis of many samples. Another option is to determine the ECF for a pure sterol that is readily available such as Cholest-5-en-3-ol (3β) (commonly known as cholesterol) or stigmasterol and apply that to the calculation of all other sterols. This technique can be problematic since determination of an ECF can be quite variable depending upon chromatographic conditions and lab-to-lab variation and because detector response changes depending on the chemical composition of a given analyte. TCF can be used to provide reliable and accurate analytical data while simplifying the preparation and data processing when analyzing samples.

A series of experiments were conducted to test the utility of the TCF and verify that they should be applied to the analysis of sterol concentrates and consumer products containing sterols. A series of solutions of the three most abundant sterols in soybean oil, stigmasterol, campesterol, and β-sitosterol were mixed with the internal standard (IS), epicoprostanol, were prepared and tested under a variety of conditions. The variables included differences in concentration of the sterols, in the temperature of the inlet, and between using split versus cool-on-column (COC) injection. The efficacy of the TCF was determined by comparing ECF for these three sterols with the TCF in terms of a percent difference, where the ideal percent difference between the TCF/ECF would be zero. When using the proper instrumental conditions and injection techniques the use of TCF were found to provide results very close to ECF.

Experimental Procedures

Calculations for Theoretical and Empirical Correction Factors

Crakse and Bannon et al. [8, 9] have invested much time and effort in developing protocols that provide the most accurate processes for analysis of FAME composition in fats and oils. They found that the most accurate system is one where the instrumental setup and operation are optimized as well as the data analysis. Instrumental optimization includes a number of factors, one factor being the sample injection including the inlet liner design, inlet temperature, volume of injection, and concentration of analytes. Optimization of data analysis includes the use of TCF as opposed to ECF, which leads to more accurate and reliable results.

Theoretical correction factors are quite easy to determine provided the compound's structure is known. First one needs to determine the number of active carbons in the compound, where active carbons are defined to be all carbons atoms with the exception of carbonyl carbons, which are not thought to respond in an FID [3]. When analyzing free sterols or stanols by GC, the standard derivatization of the hydroxyl is with a trimethylsilyl (TMS) group to improve volatility and separation resolution. The TMS group contains three carbons that meet the

above definition of active carbons, which in this work were experimentally found to contribute a response in the FID, thus they are included in the calculation for active carbons. The sum of the molecular weights of all the active carbons is then divided by the molecular weight of the entire sterol as shown in Eq. 1. This value is referred to as the TCF [4].

$$\text{Active Carbon Mass Percent} = \frac{\text{Active Carbon Molecular Weight}}{\text{Total Sterol Molecular Weight}} = \text{TCF} \quad (1)$$

In this work, the compound Cholestan-3-ol, (3α,5β) (commonly known as Epicoprostanol) was used as the internal standard (IS) and the TCF of the sterols relative to Epicoprostanol are calculated as shown in Eq. 2. Many labs currently use 5-α-Cholestane as an IS for sterol analysis, however Epicoprostanol is derivatizable due to its hydroxyl group and therefore is more similar to the compounds that are being quantitated [10]. The TCF of Epicoprostanol is divided by the TCF of each sterol of interest to give a TCF relative to the IS.

$$\frac{\text{TCF}_{\text{Epi}}}{\text{TCF}_{\text{SterolX}}} = \text{Sterol TCF Relative to IS} \quad (2)$$

A list of TCF for sterols commonly found in vegetable oils, where Epicoprostanol is the IS, is presented in Table 1. Once the TCF of all sterols of interest have been determined the amounts of each sterol can be determined from chromatographic data as shown in Eq. 3. The area of the peak of interest is multiplied by the amount of IS added and then the TCF of the sterol of interest, this value is divided by the area of the IS to give the amount of that sterol in a given sample. The percent of total sterols for a sample can then be determined by summing the amount of each individual sterol in a given sample, dividing by the sample weight and multiplying by 100.

$$\text{Amount of Sterol} = \frac{\text{Area}_{\text{Sterol}} \times \text{Amount}_{\text{IS}} \times \text{TCF}}{\text{Area}_{\text{IS}}} \quad (3)$$

Empirical correction factors were determined for the three sterol standards used for these experiments; Campesterol, Stigmasterol, and β-Sitosterol. The ECF were determined by taking the average peak area from three replicate injections and dividing by the purity adjusted amount of the sterol standard to get the empirical response factor (ERF) as shown in Eq. 4.

$$\text{Empirical Response Factor} = \frac{\text{Area}_{\text{Sterol}}}{\text{Adjusted Amount}_{\text{Sterol}}} \quad (4)$$

An ERF is calculated for both the sterol of interest and an internal standard then an empirical relative response factor (ERRF) is determined to account for response differences between the analyte of interest and the IS, as shown in Eq. 5.

$$\text{Empirical Relative Response Factor} = \frac{\text{ERF}_{\text{Sterol}}}{\text{ERF}_{\text{IS}}} \quad (5)$$

The individual ECF are then determined by taking the inverse of the ERRF [5].

Preparation of Sterol and Internal Standard Solutions

Stigmasterol (P/N S2424, Sigma Aldrich, St. Louis, MO, USA), Campesterol (P/N ASB-00003072-025, Chroma-Dex, Santa Ana, CA, USA; P/N C6700-000, Steraloids, Newport, RI), β-Sitosterol (P/N S1270, Sigma Aldrich), and Epicoprostanol (P/N C2882, Sigma Aldrich) were dissolved in toluene (P/N T291-4, Optima, Fisher Scientific, Hampton, NH, USA) at the following concentrations; 10, 5, and 2 mg/mL (actual concentrations shown in data tables). The purity (percent area) of all solutions was checked by GC/FID according to conditions below before mixing and included solvent blanks for all solvents used in these experiments. Deviations from manufacturer specifications were accounted for in the final concentrations of the solutions used for calculations.

It was imperative that an accurate purity was determined for all standards and that the purities quoted by manufacturers be disregarded, as they can often be incorrect. In order to determine the purity five replicates of the 5 mg/mL solutions of each standard (see Table 2 for exact concentrations) were prepared and analyzed via COC GC, conditions shown below. The standard preparation was as follows: pipette a 300-µL aliquot of the sterol or IS solution into a GC vial and then add 500 µL of pyridine (Pierce, Rockford, IL, USA) and 1 mL of bis (trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) (Pierce, Rockford, IL, USA). GC vials were then vortexed briefly before being analyzed by GC/FID. Purity was calculated by determining the total peak area for all peaks eluting in the chromatogram and expressing the peak area of the standard as a percentage of that total area. Blanks were run to eliminate the inclusion of any contaminates from the solvents used to prepare the standard solutions.

To compare empirical results to theoretical predictions, solutions were made up at three different concentrations with approximately equal amounts of a given sterol along with the IS. This was done by pipetting 5 mL of a sterol solution and 5 mL of IS solution at similar concentrations into a scintillation vial using 5-mL Class A volumetric pipettes. The sterol/IS solution was then gently mixed and prepared for GC analysis by taking a 300-µL aliquot of the sterol/IS mix into a GC vial and then adding 500 µL of pyridine (Pierce, Rockford, IL) and 1 mL of bis (trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) (Pierce). GC vials were then vortexed briefly before being

Table 1 Theoretical correction factors calculated for sterols commonly found in vegetable oils relative to Epicoprostanol as the internal standard

Common sterol name	Sterol name (Chemical Abstracts, CA)	CA number	Empirical formula	Theoretical correction factor (TCF)
Epicoprostanol	Cholestan-3-ol, (3α,5β)	516-92-7	$C_{27}H_{48}0$	1.0000
Cholesterol	Cholest-5-en-3-ol (3β)	57-88-5	$C_{27}H_{46}0$	0.9956
Brassicasterol	Ergosta-5,22-dien-3-ol, (3β,22E)	474-67-9	$C_{28}H_{46}0$	0.9887
Ergosterol	Ergosta-5,7,22-trien-3-ol, (3β,22E)	57-87-4	$C_{28}H_{44}0$	0.9845
Desmosterol	Cholesta-5,24-dien-3-ol, (3β)	313-04-2	$C_{27}H_{44}0$	0.9913
Δ7-Brassicasterol	Ergosta-7,22-dien-3-ol, (3β,22E)	17608-76-3	$C_{28}H_{46}0$	0.9887
24-Methylenecholesterol	Ergosta-5,24(28)-dien-3-ol, (3β)	474-63-5	$C_{28}H_{46}0$	0.9887
Campestanol	Ergostan-3-ol, (3β,5α,24R)	474-60-2	$C_{27}H_{50}0$	0.9972
Campesterol	Ergost-5-en-3-ol, (3β,24R)	474-62-4	$C_{28}H_{48}0$	0.9930
β-Sitosterol	Stigmast-5-en-3-ol, (3β)	83-46-5	$C_{29}H_{50}0$	0.9905
Stigmasterol	Stigmasta-5,22-dien-3-ol, (3β,22E)	83-48-7	$C_{29}H_{48}0$	0.9864
Δ22-Stigmastenol	Stigmast-22-en-3-ol, (3β,5α,22E,24ξ)	65494-30-6	$C_{29}H_{50}0$	0.9905
No common name	Stigmasta-7,22,24(28)-trien-3-ol, (3β,5α,22E,24Z)	178275-60-0	$C_{29}H_{46}0$	0.9823
No common name	Ergost-7-en-3-ol, (3β,24R)	70095-94-2	$C_{28}H_{48}0$	0.9930
Lanosterol	Lanosta-8,24-dien-3-ol, (3β)	79-63-0	$C_{30}H_{50}0$	0.9841
α-Amyrin	Urs-12-en-3-ol, (3β)	638-95-9	$C_{30}H_{50}0$	0.9841
β-Amyrin	Olean-12-en-3-ol, (3β)	559-70-6	$C_{30}H_{50}0$	0.9841
Sitostanol	Olean-3-ol, (3β,5α)	83-45-4	$C_{29}H_{52}0$	0.9925
Clerosterol	Stigmasta-5,25-dien-3-ol, (3β,24S)	2364-23-0	$C_{29}H_{48}0$	0.9864
Δ7-Stigmasterol	Stigmasta-5,7,22-trien-3-ol, (3β)	481-19-6	$C_{29}H_{46}0$	0.9864
Δ5-Avenasterol	Stigmasta-5,24(28)-dien-3-ol, (3β)	18472-36-1	$C_{29}H_{48}0$	0.9864
Δ7-Sitosterol	Stigmasta-5,7-dien-3-ol, (3β)	521-04-0	$C_{29}H_{48}0$	0.9905
Δ22-Stigmastenol	Stigmasta-7,24(28)-dien-3-ol, (3β)	17105-79-2	$C_{29}H_{48}0$	0.9864
No common name	Stigmast-7-en-3-ol, (3β,5α)	521-03-9	$C_{29}H_{50}0$	0.9905
Δ7-Avenasterol	Stigmasta-7,24(28)-dien-3-ol, (3β,5α,24Z)	23290-26-8	$C_{29}H_{48}0$	0.9864
Citrostadienol	Stigmasta-7,24(28)-dien-3-ol, 4-methyl-, (3β,4α,5α,24Z)	474-40-8	$C_{30}H_{50}0$	0.9864
Fucosterol	Stigmasta-5,24(28)-dien-3-ol, (3β,24E)	17605-67-3	$C_{29}H_{48}0$	0.9864

Table 2 Summary of standard solutions prepared to for the determination of sterol purities

Sterol	Concentration (mg/mL)	Average purity	Standard deviation	Manufacturer
Stigmasterol	5.1	92.6	0.05	Sigma-Aldrich
Campesterol	4.4	77.2	0.29	Steraloids
β-Sitosterol	5.5	94.0	0.02	Sigma-Aldrich
Campesterol	5.5	98.9	0.08	ChromaDex
Epicoprostanol	5.1	99.8	0.02	Sigma-Aldrich

analyzed by GC/FID. All samples were prepared and run in triplicate under each of the different condition to check reproducibility and error.

Chromatographic Conditions

Split Injection GC Conditions

The TMS ethers of the sterols were analyzed by split gas chromatography (GC) with a non-polar column stationary phase (DB^{TM}-5, 30 m × 0.25 mm × 0.25 μm, Agilent Technologies, Santa Clara, CA, USA) coupled to a flame ionization detector (FID) using a 6850 GC equipped with an autosampler (Agilent Technologies, Santa Clara, CA, USA). The temperature program was 200–300 °C at 3 °C/min (11.67 min) [11]. Hydrogen was the carrier gas, and inlet pressure was 9 psi in the constant flow mode with a flow rate of 0.8 mL/min. Samples were evaluated at two different split ratios 1:35 and 1:175. Injection volume was 1 μL and the injector and detector temperatures were both

325 °C. The detector gas flows were set to 450 mL/min for air, 40 mL/min for hydrogen and 45 mL/min for the nitrogen make up gas.

Cool-on-Column GC Conditions

The TMS ethers of the sterols were also analyzed by COC gas chromatography (GC) with a high temperature non-polar column stationary phase (DB^{TM}-5HT, 15 m × 0.25 mm × 0.10 μm, Agilent Technologies, Santa Clara, CA) coupled to a flame ionization detector (FID) using a 6890 GC equipped with an autosampler (Agilent Technologies). The temperature program was 110–140 °C at 3 °C/min, 140–340 °C at 10 °C/min, hold 13.80 min. Hydrogen was the carrier gas, and inlet pressure was 6.7 psi at a temperature of 110 °C in the constant flow mode with a flow rate of 1.25 mL/min. Injection volume was 0.5 μL and the detector temperature was 370 °C. Oven tracking was used for the injector temperature. The detector gas flows were set to 450 mL/min for air, 40 mL/min for hydrogen and 40 mL/min for the nitrogen make up gas.

TMS Signal Verification

In order to determine if the carbons on the TMS group would be detected by an FID a simple experiment was performed where three standard solutions of campesterol, stigmasterol, and β-sitosterol each were made up at a concentration of approximately 1 mg/mL. For each of these standards two sets of triplicate samples were prepared and injected onto the GC following the same conditions as described above for the split injection GC. The first set of triplicate standards were derivatized following our protocol discussed above. The second set of triplicate standards was not derivatized and 1.5 mL of toluene was added to the standard solution in the GC vial to account for dilution differences. The average peak area of the derivatized sterol solution was then compared to the average peak of the same underivatized sterol solution.

In addition, a standard of trimethyl chlorosilane (TMCS) (Pierce Chemical) was injected onto a GC/FID to determine if it gave a signal. The same split injection GC conditions indicated above were used for this injection. The identity of this peak was confirmed by GC/MS where the TMCS standard was chromatographed with a non-polar column stationary phase (DB^{TM}-5, 30 m × 0.25 mm × 0.25 μm, Agilent Technologies). The temperature program was 200–300 °C at 3 °C/min (11.67 min) [11]. Helium was the carrier gas, and inlet pressure was 24.2 psi in the constant flow mode at 1.6 mL/min with a split ratio of 10:1. The mass spectra were collect using an Agilent quadrapole mass spectrometer with source temperature of 230 °C and voltage of 70 eV. A full scan of was performed from 15 to 800 Da. Collected spectra were matched to spectra in the NIST mass spectral database, version 2.0a, July 1 2002, and with an in-house spectral library.

Statistical Analysis

A statistical analysis of the data was done to show whether or not the differences between experiments are significant or not. The *T* test function embedded in Excel 2000 (version 9.0.8968 SP-3, Microsoft Corp. Remond, WA, USA) was used to compare average peak area differences. This function returns a *p*-value from two arrays and a paired, one-tailed distribution was used for our data. The *F*-test function embedded in Excel 2000 was used to determine the significance of variation within samples. This function returns a *p*-value from two arrays based on the variation between data. For both the *T*-test and the *F*-test the 95% confidence interval was chosen where a *p*-value of less then 0.05 was considered to be a significant difference.

Results and Discussion

Before discussing the results of the ECF/TCF percent differences it was important to show that the three methyl groups on the TMS group do indeed give a response in an FID and should be included in the TCF calculation as previous authors have stated that this group does not contribute to the FID signal [12]. Table 3 shows the peak area data collected from a series of injections of derivatized and underivatized campesterol, stigmasterol, and β-sitosterol standard solutions. It can be seen in Table 1 that the average peak area for the derivatized sterols is higher than the average peak area for the underivatized sterols. A statistical analysis of the average peak areas for all three sterols using the *T*-test returned a *p*-value of 0.002 indicating the differences in the peak areas of underivatized sterols and derivatized sterols are significant. This shows that the extra carbons on the TMS group do indeed contribute to the FID signal and need to be accounted for in the determination of TCF.

A second experiment was conducted where a TMCS standard was injected onto the GC to see if a signal was observed. There was a strong peak observed in the chromatogram at 1.68 min that would be a result of the combustion of carbon containing molecules. The identity of this peak as TMCS was also confirmed by mass spectrometry (MS) where a large peak eluting at 1.55 min had a mass spectrum with a base peak at $m/z = 92$, a molecular ion at $m/z = 108$ that both exhibited "a + 2" ions typical of chlorine containing compounds [13]. Other major fragments in the spectrum included $m/z = 73$, 65, and 63.

Table 3 Summary of peak area data collected for standard solutions of derivatized and underivatized sterols

Sterol	Peak area			Average peak area	Standard deviation	Concentration (mg/mL)
	$n = 1$	$n = 2$	$n = 3$			
Derivatized sterols						
Campesterol	304.0	296.5	292.1	297.5	6.02	1.0
β-Sitosterol	285.9	278.0	280.5	281.5	4.04	1.0
Stigmasterol	278.9	282.4	274.0	278.4	4.22	1.0
Underivatized sterols						
Campesterol	221.9	214.9	212.1	216.3	5.05	1.0
β-Sitosterol	203.0	192.3	191.0	195.4	6.59	1.0
Stigmasterol	207.9	206.8	211.0	208.6	2.18	1.0

A comparison of the collected spectrum to the NIST database spectra also provided a positive match. Thus it was determined that the TMS carbons do contribute to the FID signal and should be included as part of the TCF calculation.

The first two data sets, shown in Tables 4 and 5, show the experimentally determined ECF, the TCF, and the average percent difference in the ECF/TCF for the three different sterols using split injections of 1:35 and 1:175. Statistical analysis of the data in Tables 4 and 5 indicate that there is no significant difference between the calculated ECF value for the different split ratios for all three sterols. The p-value determined comparing the variance in data between Table 4 and 5 was 0.24. Taking a 95% confidence interval (CI) this value is well above 0.05 and indicates that the variation between the two split injection techniques is not statistically significant, which is in line with our hypothesis that there is not a significant difference determination of the ECF by different split ratios.

The data in Tables 4 and 5 also show differences in the ECF values determined for the same sterol as concentrations of the sterol decreases. This is especially noticeable for campesterol, which shows a huge deviation in ECF value at the lowest concentration. Statistical analysis (T-test) of the ECF values calculated from the standards at different concentrations indicates that this hypothesis is true especially between the $\sim$10-mg/mL solutions versus the $\sim$2-mg/mL solutions.

One way to compare ECF values to TCF values is to look at the percent difference between the measured value and the calculated value. The ideal situation would be that the percent difference is zero. The data in Table 4 for the 1:35 split injection shows that the average ECF/TCF percent difference for stigmasterol and β-sitosterol were close to each other and relatively close to zero. A very similar result was observed in the data collected using the 1:175 split, which is shown in Table 5. There was an inconsistency, however, in the ECF values for campesterol that may be attributed to the fact that the campesterol standard purity was only 77.2% (normalized area percent) whereas the stigmasterol and β-sitosterol purities were 92.6 and 94.0%, respectively, as determined by GC/FID. The more impurities found in a given standard the further the ECF/TCF difference was from ideal (zero). This comes directly from the difference in the mass of a standard that is weighed on a balance for a given solution and the actual

Table 4 Summary of ECF and TCF determined for standard sterol solutions using 1:35 split injection

Sterol	Average purity	Concentration (mg/mL)	Average ECF	Standard deviation	TCF	Average %ECF/TCF difference
Stigmasterol	92.6	9.3	1.0242	0.0017	0.9864	3.84
Campesterol	77.2	6.7	1.1417	0.0024	0.9930	14.97
β-Sitosterol	94.0	10.3	1.0333	0.0014	0.9905	4.32
Stigmasterol	92.6	4.7	1.0129	0.0035	0.9864	2.69
Campesterol	77.2	3.4	1.1436	0.0021	0.9930	15.17
β-Sitosterol	94.0	5.1	1.0203	0.0226	0.9905	3.01
Stigmasterol	92.6	1.9	1.0578	0.0037	0.9864	7.23
Campesterol	77.2	1.7	1.4591	0.0116	0.9930	46.94
β-Sitosterol	94.0	2.0	1.0632	0.0064	0.9905	7.34

Table 5 Summary of ECF and TCF determined for standard sterol solutions using 1:175 split injection

Sterol	Average purity	Concentration (mg/mL)	Average ECF	Standard deviation	TCF	Average %ECF/TCF difference
Stigmasterol	92.6	9.3	1.0235	0.0028	0.9864	3.76
Campesterol	77.2	6.7	1.1321	0.0010	0.9930	14.01
β-Sitosterol	94.0	10.3	1.0293	0.0018	0.9905	3.92
Stigmasterol	92.6	4.7	1.0115	0.0014	0.9864	2.54
Campesterol	77.2	3.4	1.1334	0.0037	0.9930	14.13
β-Sitosterol	94.0	5.1	1.0202	0.0036	0.9905	3.00
Stigmasterol	92.6	1.9	1.0549	0.0145	0.9864	6.94
Campesterol	77.2	1.7	1.4475	0.0066	0.9930	45.77
β-Sitosterol	94.0	2.1	1.0440	0.0096	0.9905	5.40

Table 6 Summary of ECF and TCF determined for standard sterol solutions using cool-on-column injection

Sterol	Average purity	Concentration (mg/mL)	Average ECF	Standard deviation	TCF	Average %ECF/TCF difference
Stigmasterol	92.6	9.3	0.9894	0.0002	0.9864	0.31
Campesterol I	77.2	6.7	1.1059	0.0004	0.9930	11.37
Campesterol II	98.9	10.8	1.0707	0.0007	0.9930	7.82
β-Sitosterol	94.0	10.3	0.9819	0.0007	0.9905	−0.87
Stigmasterol	92.6	4.7	1.0043	0.0496	0.9864	1.82
Campesterol I	77.2	3.4	1.0989	0.0002	0.9930	10.66
Campesterol II	98.9	5.4	1.0592	0.0008	0.9930	6.66
β-Sitosterol	94.0	5.1	0.9737	0.0004	0.9905	−1.69
Stigmasterol	92.6	1.9	0.9968	0.0002	0.9864	1.06
Campesterol I	77.2	1.7	1.4119	0.0071	0.9930	42.19
Campesterol II	98.9	2.2	1.0823	0.0016	0.9930	9.00
β-Sitosterol	94.0	2.1	0.9814	0.0017	0.9905	−0.92

amount of that mass that is the standard of interest. Determining the percent purity of each standard and factoring that into the actual mass of the analyte should account for this factor. However, as standards become less pure the likelihood of missing impurities increases and larger errors in the ECF determination will occur. In addition, this further shows larger problems when using ECFs as they can be time consuming to determine, high purity standards are needed, and influence from experimental errors can cause lose in precision and accuracy of sterol measurements.

The next data set, shown in Table 6, gives the ECF and TCF data for four different sterols solutions using COC injection. There is an additional data set that was collected from a more pure source of campesterol (98.9%, Campesterol II) that was found through a different vendor. The variability between ECF determinations was analyzed by looking at the *p*-values calculated the standard deviations between Tables 4 and 6, and Tables 5 and 6, which were 0.05 and 0.003, respectively. According to our 95% CI these values indicate that the consistency of the ECF determined using COC injection are statistically significant from the split injection based ECF. The 1:35 split ratio gave a *p*-value of 0.05 compared to the COC, however, it should be noted that the variance in one of the samples is much higher and could be due to an outlying data point. The data from the larger split ratio gave a *p*-value of 0.003, which is clearly a significant difference, which statistically shows COC injection is the preferred technique to use when determining ECF and shows another weakness of ECF determinations.

As was observed with the split injection data the percent differences for Stigmasterol and β-sitosterol were close to each other and very close to zero, which is ideal. In fact, the percent difference is much closer to zero than it was with either of the split injections. Consistent with the

results of the split injection experiments the percent difference for Campesterol I was not close to the other two sterols, is relatively far from zero, and appears to again show concentration dependence. Additionally in Table 6 there is Campesterol II, while not quite ideal it is a little closer to the other two sterols, closer to zero, and does not show a significant concentration dependence. This indicates that purity indeed plays a role in ECF determination but there may be other factors that have to be accounted for, such as better instrument optimization as indicated by Craske and Bannon [8]. This data shows that COC injection also provides a more ideal injection and eliminates any variability that might be introduced by the electronic pressure control (EPC) unit used in split injectors. It also illustrates how different laboratories purchasing standards from different vendors could have inconsistencies in their ECF determinations that would be reflected in their sterol calculations.

This work explored the use of theoretical correction factors (TCFs) in the analysis of sterol concentrates. TCF were found to be much simpler to work than the empirical correction factors (ECFs), especially for sterols where high purity standards can be difficult or impossible to obtain. It remains unclear why the % ECF/TCF difference is not closer to zero for the case of the split injections for the campesterol standard and additional experiments may be need to determine this. The main issues revolve around the optimization of the GC as discussed by Craske and Bannon [8] such as the effects of different injection types, different inlet liners, as well as the influence of detector gas flow parameters have on ECF determinations Use of TCF in analysis of sterol concentrates and sterol containing consumer products can significantly improve method reproducibility and accuracy.

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